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Features of a donor vector.

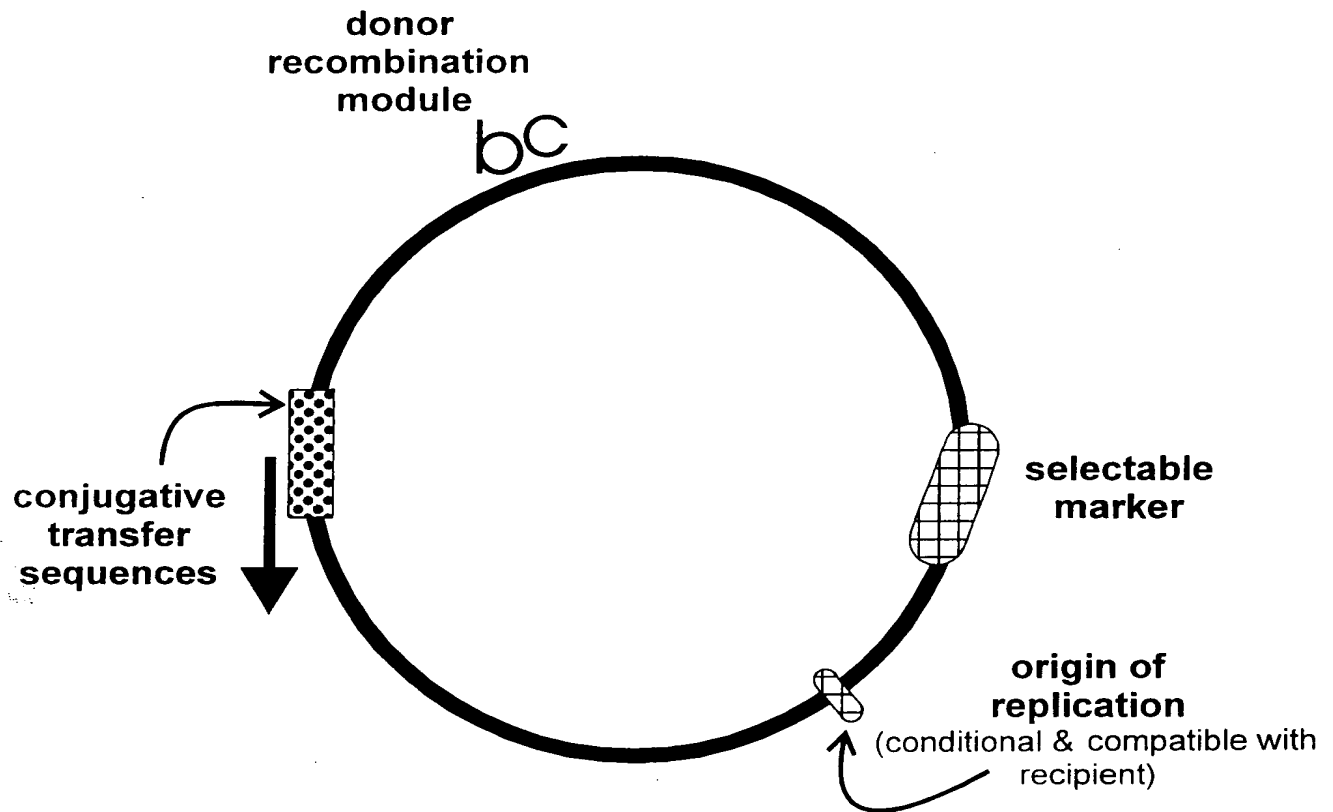
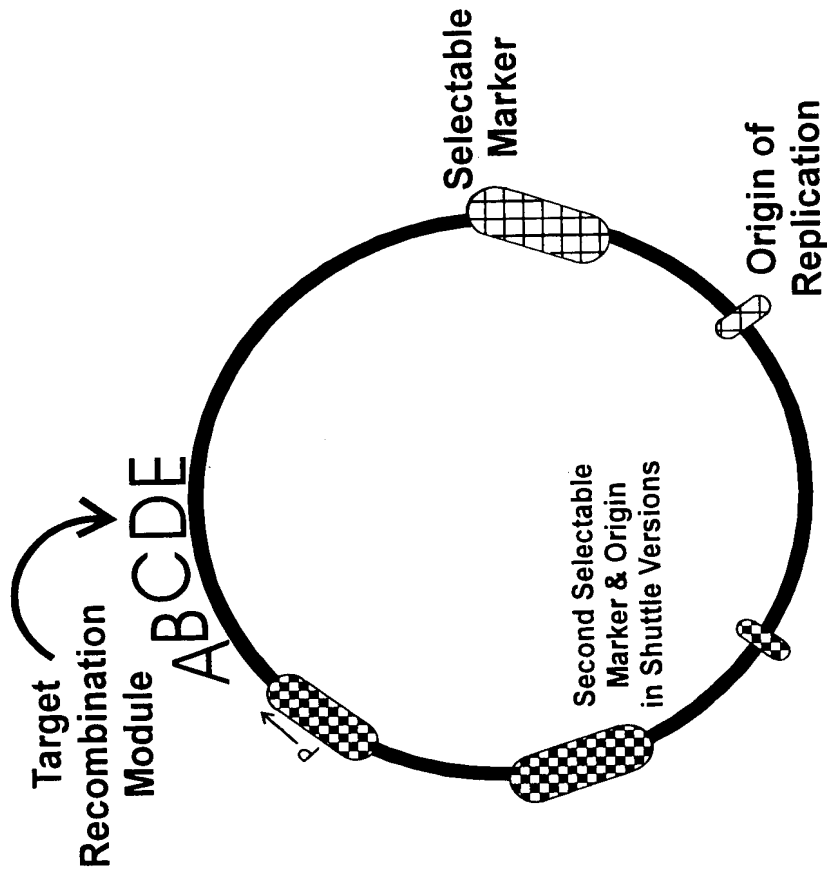


FIG.1

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Features of a target vector.



Additional Features of DGA

Galk expression is lethal on galactose in a gal/E background. & The insert is negatively-selectable.

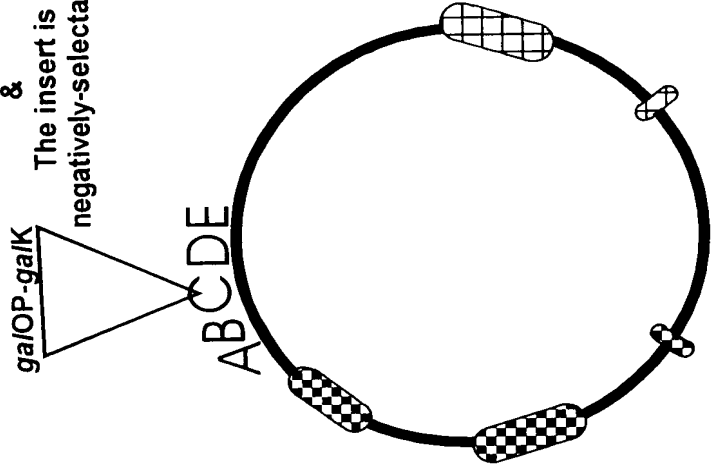
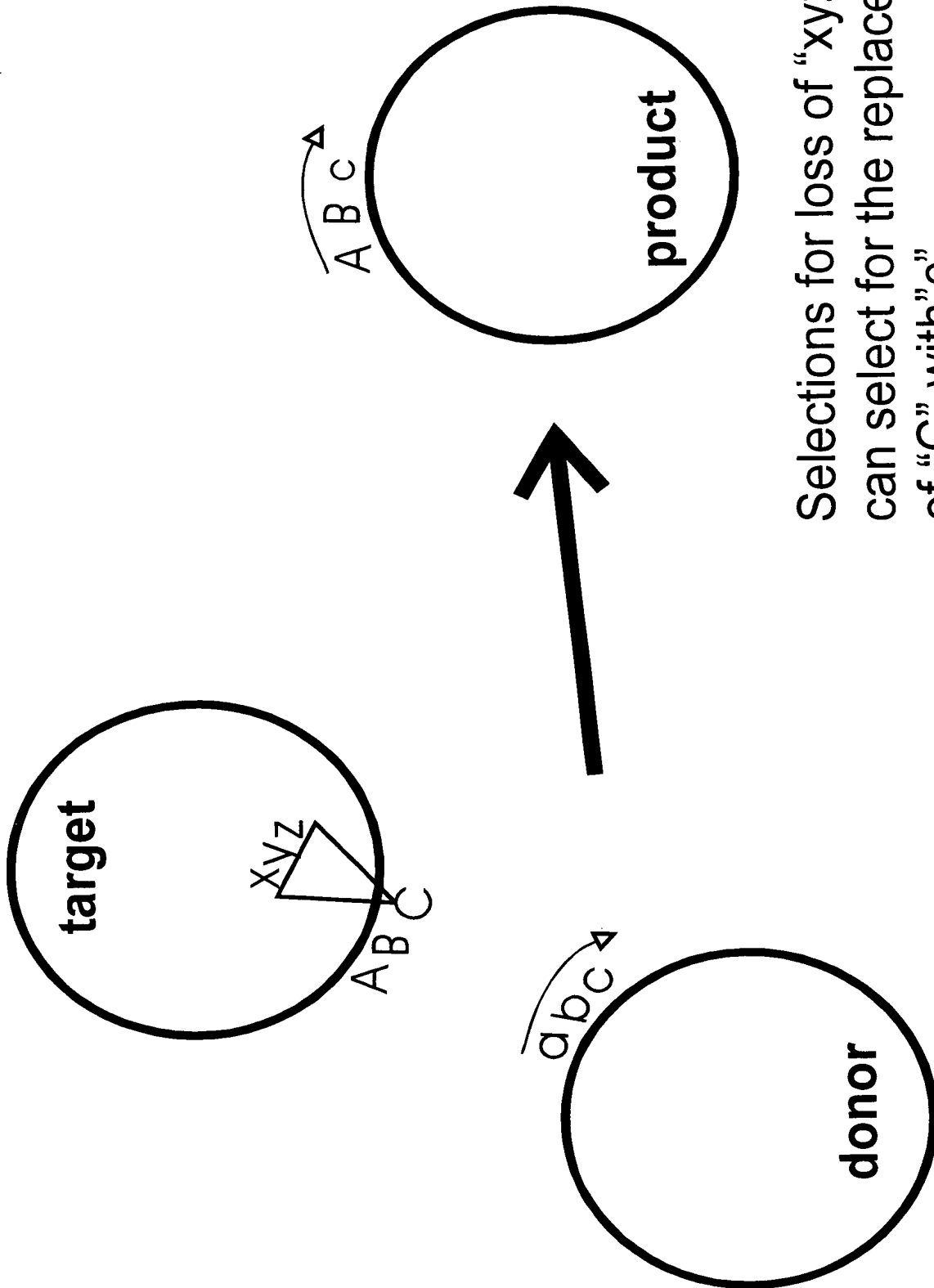


FIG.2

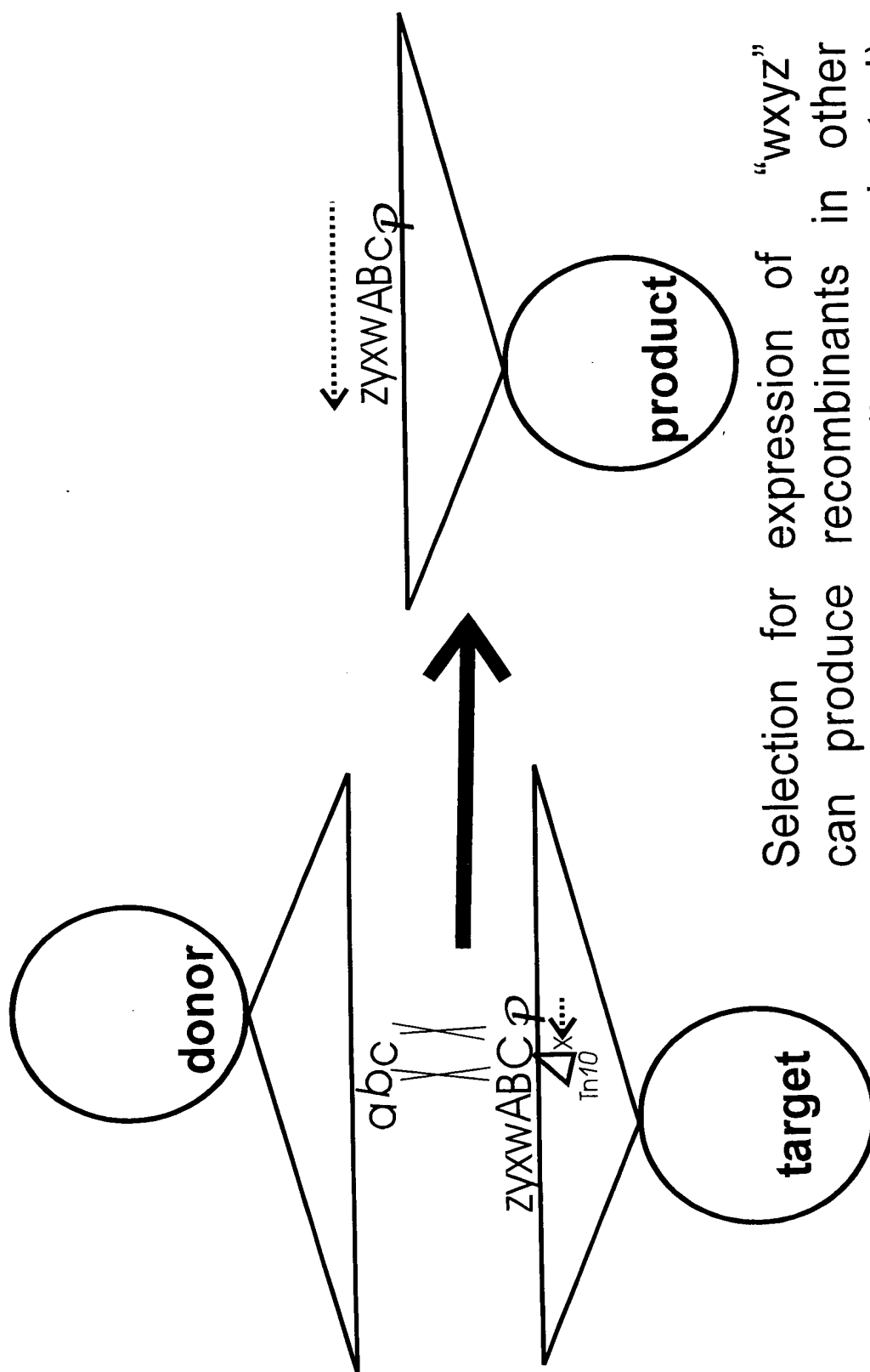
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Selections for loss of "xyz"
can select for the replacement
of "C" with "c".

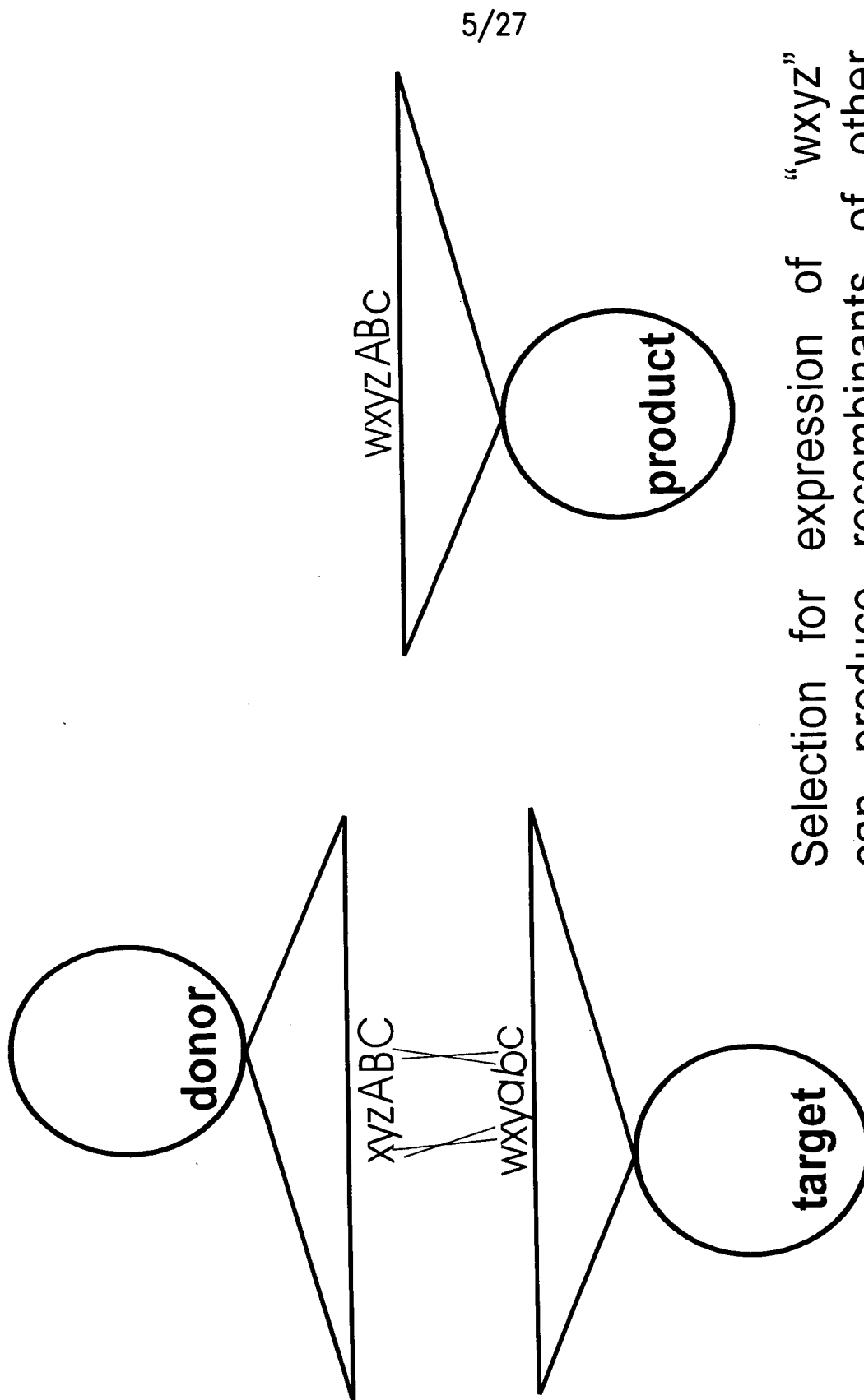
FIG.3

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Selection for expression of "wxyz" can produce recombinants in other physically linked (but unselected) homologous sequences, such as, "ABC", in this example through removal of the polar Tn10 sequences.

FIG.4



Selection for expression of "wxyz" can produce recombinants of other physically linked homologous sequences, such as, "Abc" in the illustration.

FIG.5

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Directed Gene Assembly applied to mutagenesis

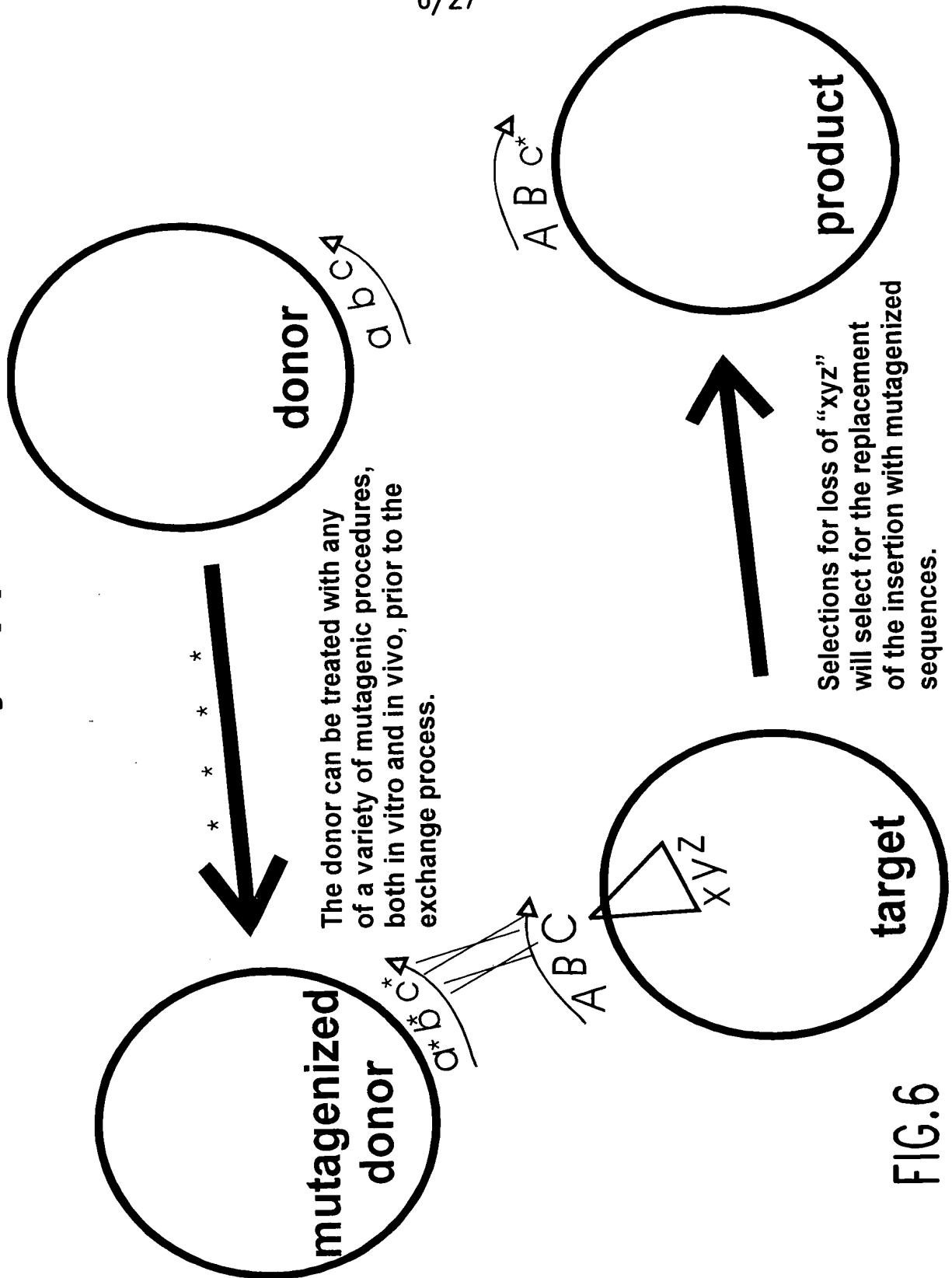
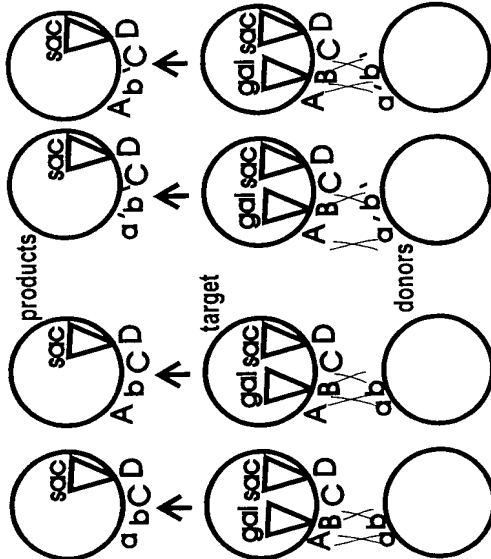


FIG.6

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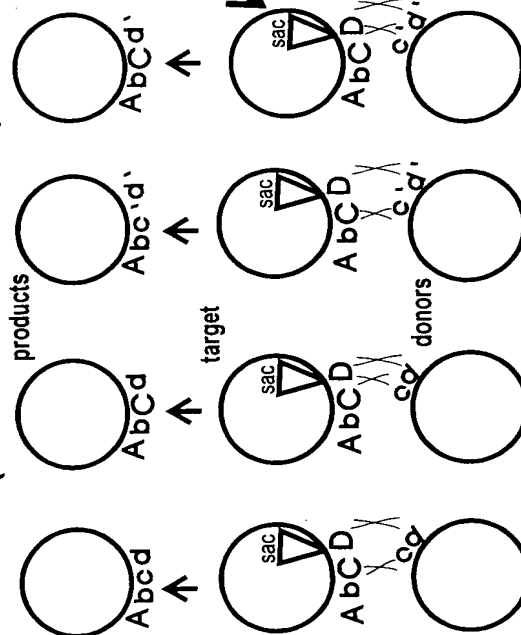
First Product Series (galactose resistant)

Products of the first galactose resistant selected reaction still contain the sucrose insert disrupting the target.



Second Product Series (sucrose resistant)

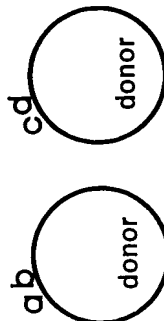
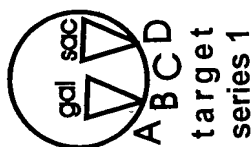
After the second sucrose resistance selection intact genes are reconstructed.



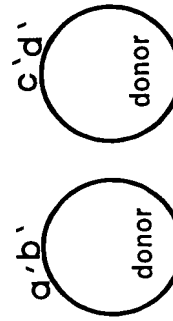
Products of the first reaction become the substrates of the second. Reactions with "AbCD" product are shown.

Substrates

Target gene with family member #1 (complete) with two recombination-targeting inserts in target vector.



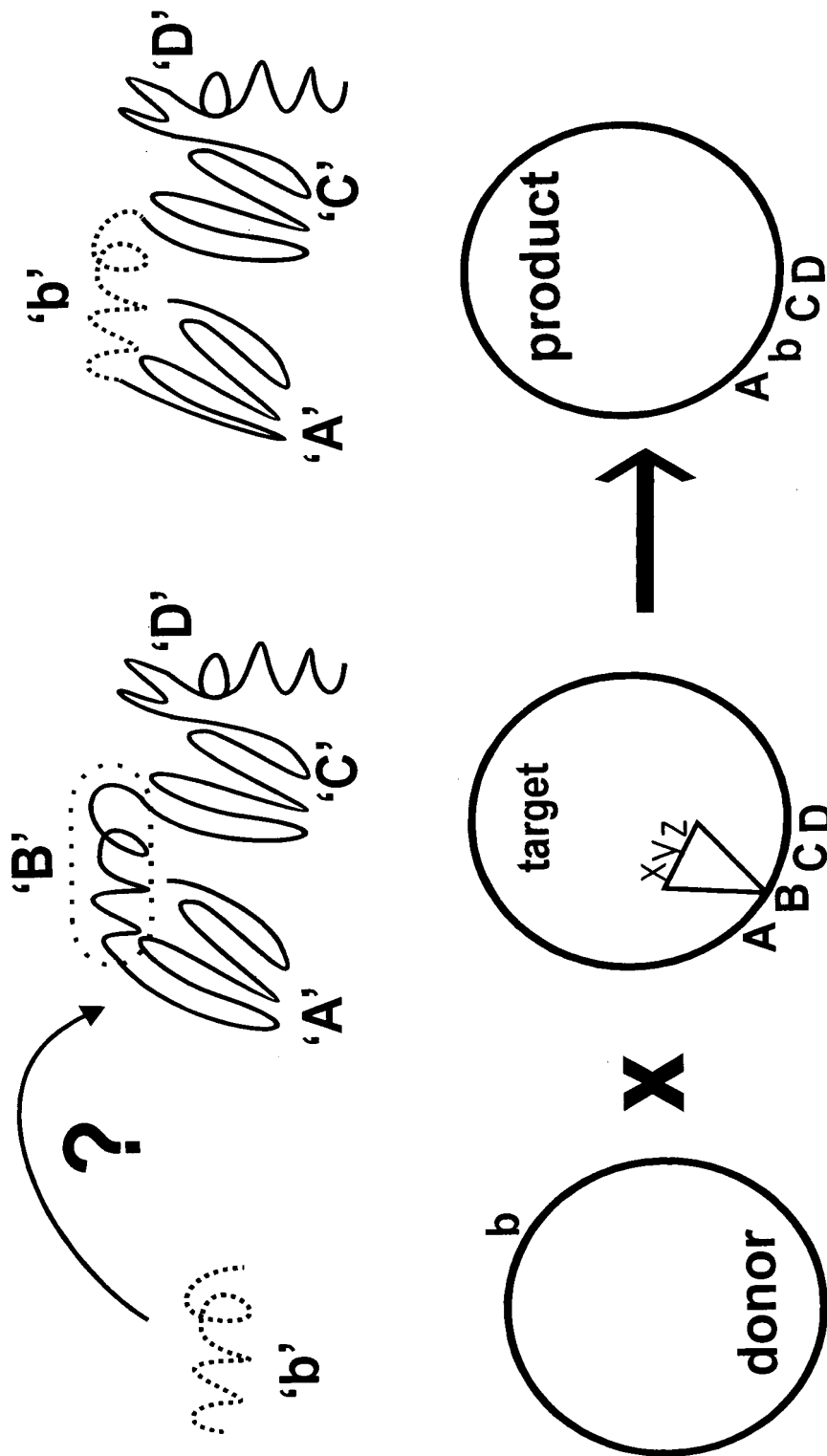
Fragments of family member #2 in donor vector library.



Fragments of family member #3 in donor vector library.

FIG. 7

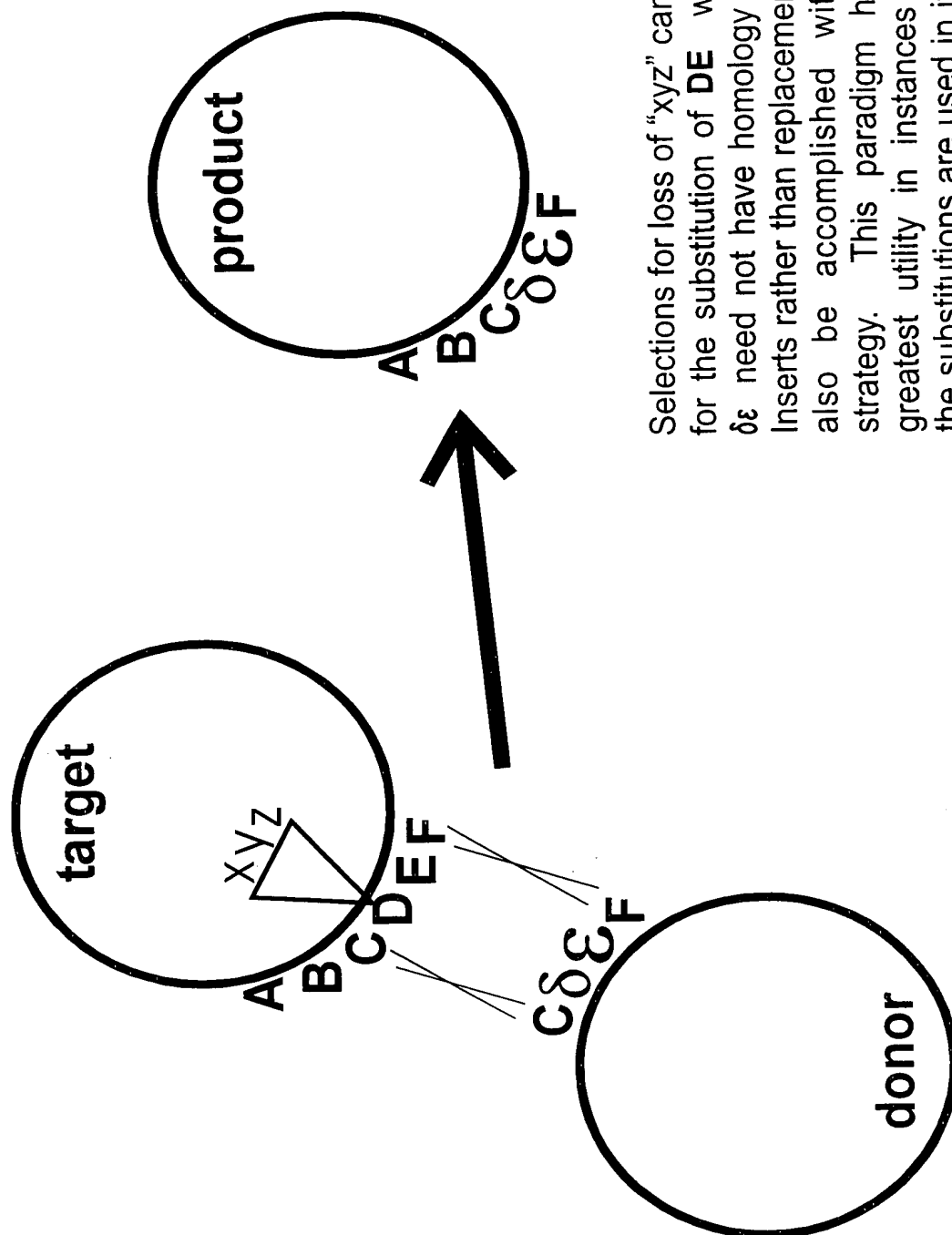
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An assay of product function will assess 'b's ability to substitute for 'B'. This paradigm executed with many target proteins and donor domains (and motifs) will identify a library of protein building blocks.

FIG.8

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Selections for loss of "xyz" can select for the substitution of **DE** with $\delta\epsilon$. $\delta\epsilon$ need not have homology to DE. Inserts rather than replacements can also be accomplished with this strategy. This paradigm has the greatest utility in instances where the substitutions are used in iterative combinatorial processes.

FIG.9

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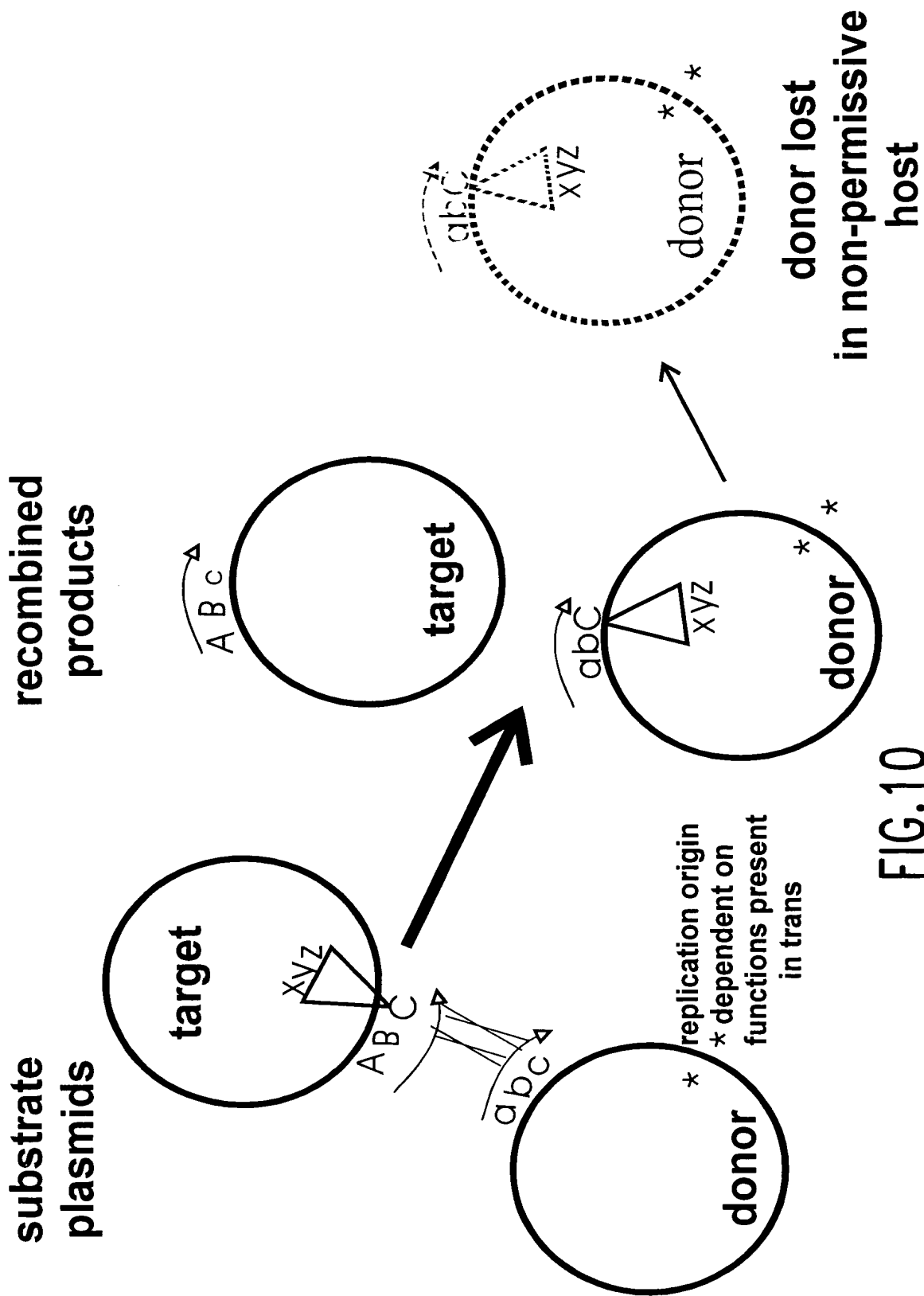
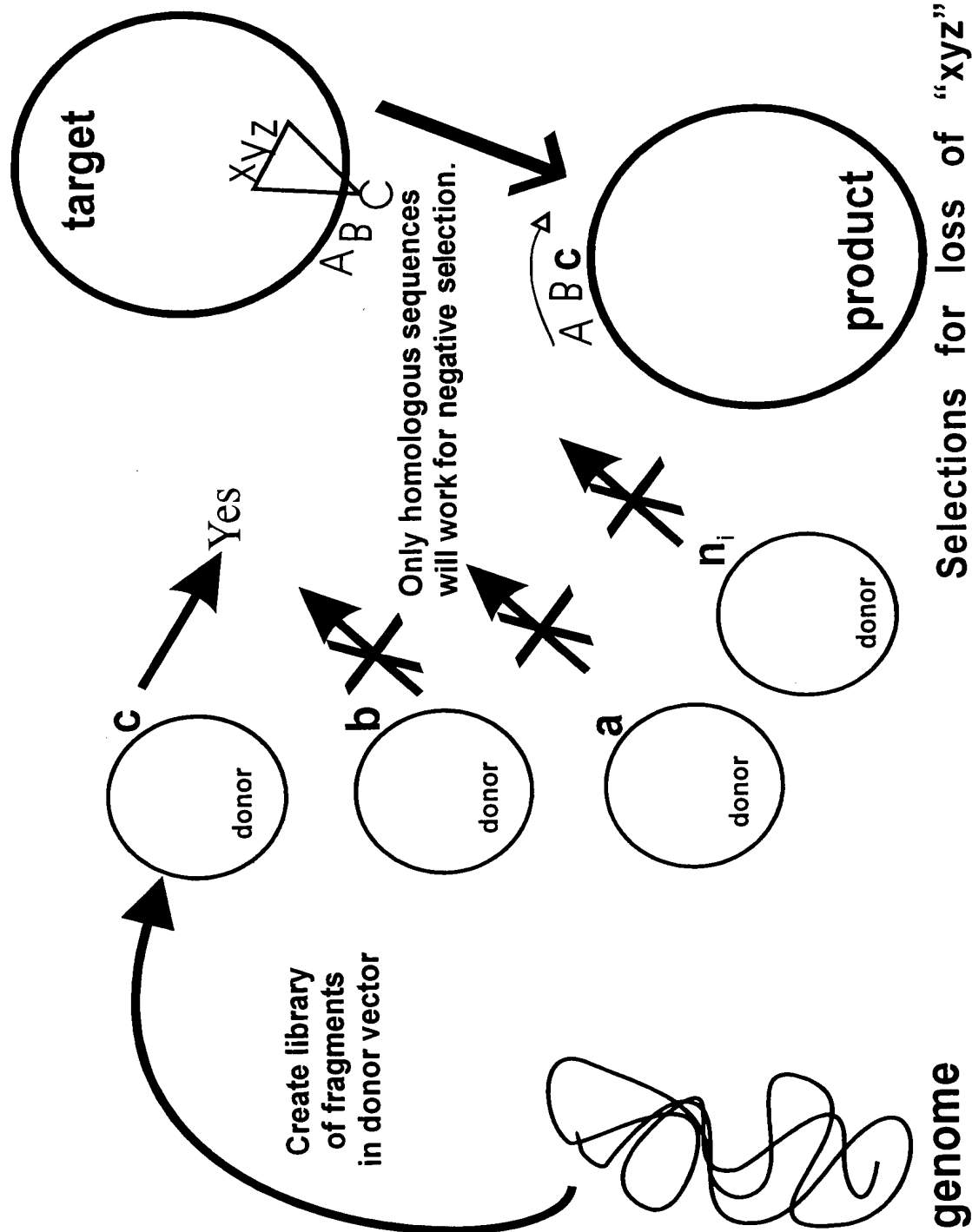


FIG.10

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Selections for loss of "xyz" can select homologous sequences from a complex a donor cloned library.

FIG. 11

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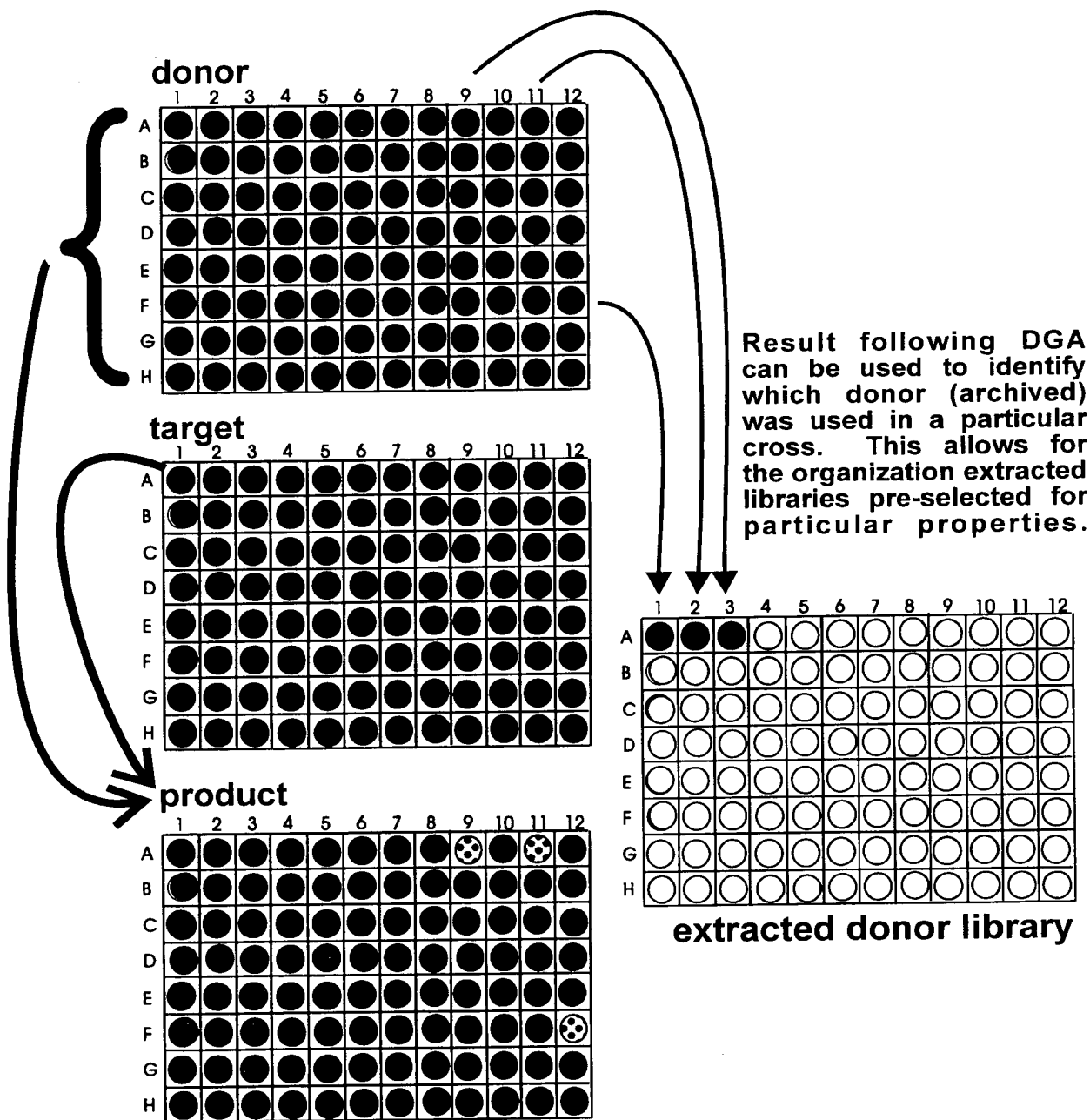
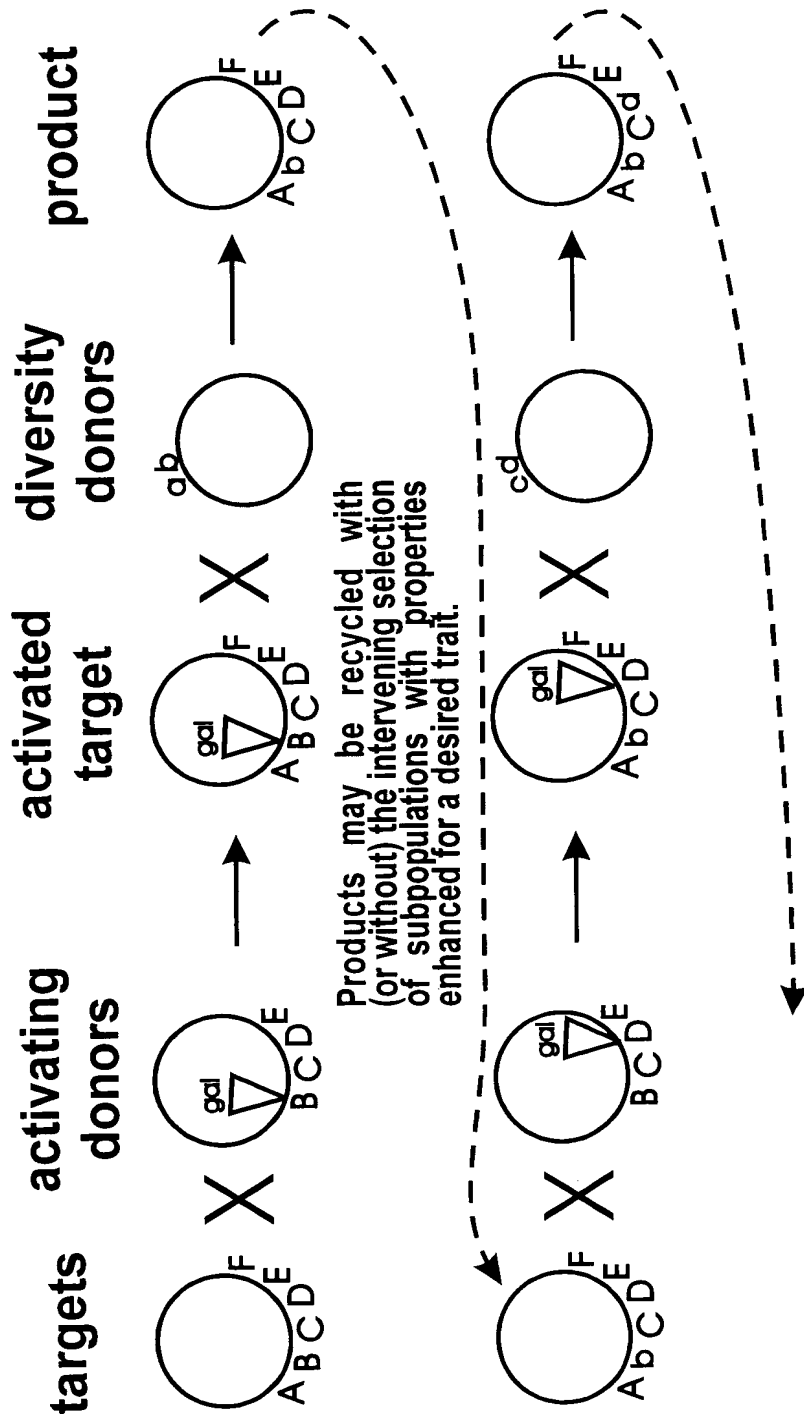


FIG. 12

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DGA Activation DGA Diversity



With the product recycled via a DGA activation step iterative cycles are possible. Iterative cycles will allow huge numbers of combinatorial arrangements to be produced from a relatively small defined set of substrates in a systematic process.

FIG. 13

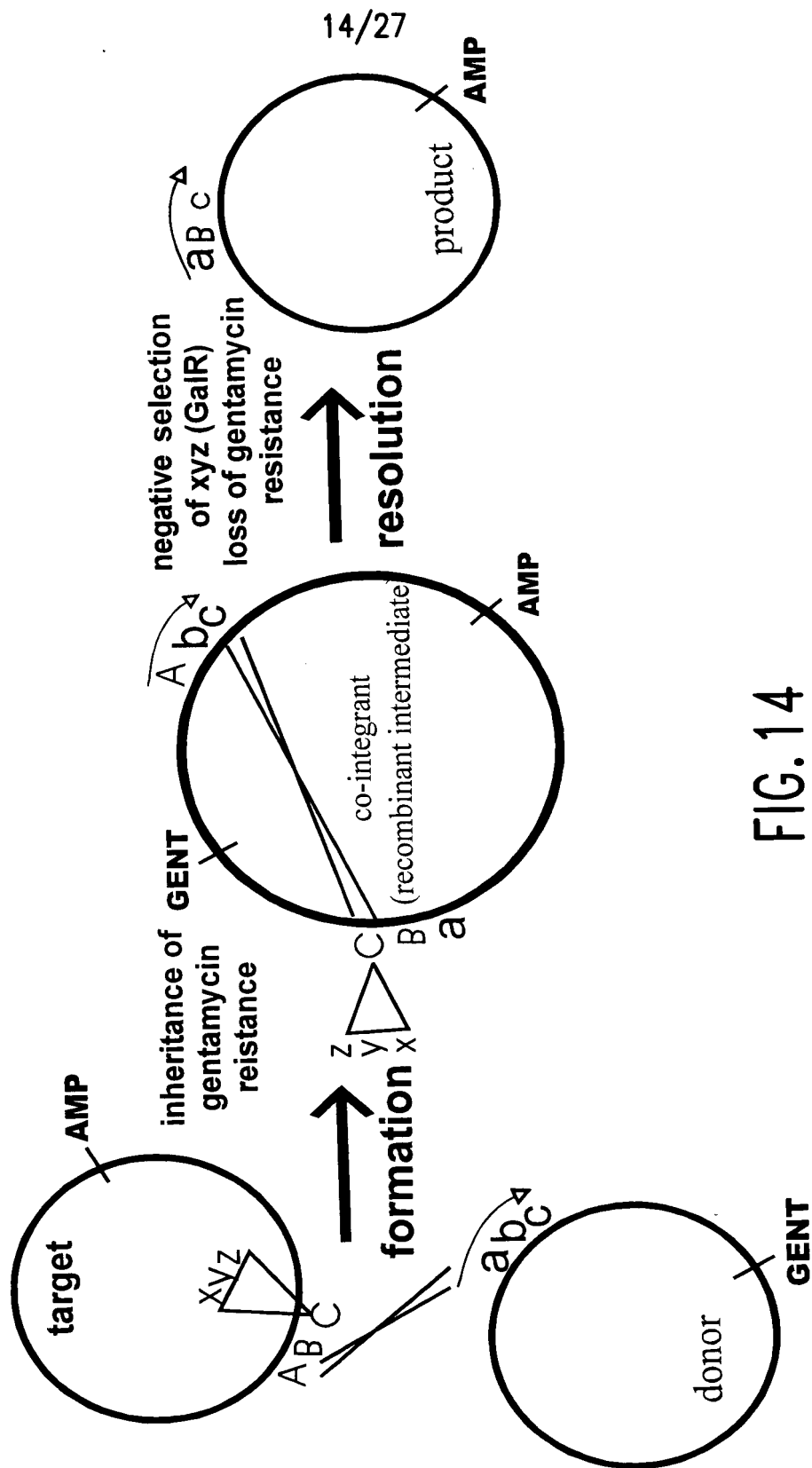


FIG. 14

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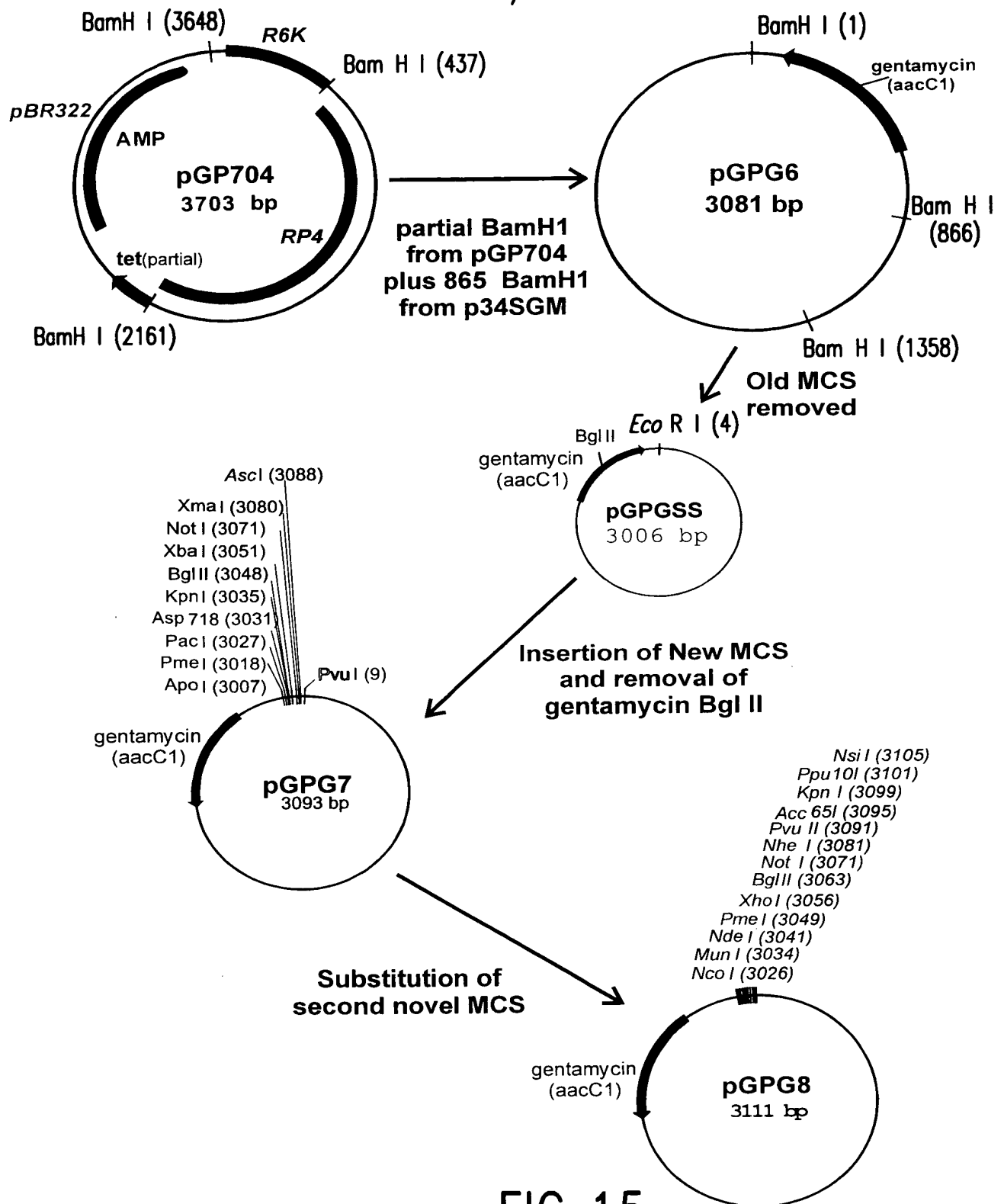


FIG. 15

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+1 I K A D K V Q A Q G F K G A N V K V A V L
1 ATTAAAGCG GACAAAGTG CAGGCTCAA GGCTTTAAG GGAGCGAAT GTAAAAGTA GCCGTCCTG

+1 D T G I Q A S H P D L N V V G G A S F V A
64 GATACAGGA ATCCAAGCT TCTCATCCG GACTTGAAC GTAGTCGGC GGAGCAAGC TTTGTGGCT

+1 G E A Y N T D G N G H G A H V A G T V A A
127 GGCGAAGCT TATAACACC GACGGCAAC GGACACGGC GCACATGTT GCCGGTACA GTAGCTGCG

+1 L D N T T G V L G V A P S V S L Y A V K V
190 CTTGACAAT ACAACGGGT GTATTAGGC GTTGCGCCA AGCGTATCC TTGTACGCG GTTAAAGTA

+1 L N S S G S G S Y S G I V S G I E W A T T
253 CTGAATTCA AGCGGAAGC GGATCATAC AGCGGCATT GTAAGCGGA ATCGAGTGG GCGACAACA

+1 N G M D V I N M S L G G A S G S T A M K Q
316 AACGGCATG GATGTTATC AATATGAGC CTTGGGGGA GCATCAGGC TCGACAGCG ATGAAACAG

+1 A V D N A Y A K G V V V V A A A G N S G S
379 GCAGTCGAC AATGCATAT GCAAAGGG GTTGTGCTT GTAGCTGCA GCAGGGAAC AGCGGATCT

DNA Sequence 5A20 *B.licheniformis* isolate

+1 I K A P A L H S Q G Y T G S N V K V A V I
1 ATTAAAGCG CCGGCTCTT CACTCTCAA GGCTACACA GGTTCTAAC GTAAAAGTA GCCGTAATT

+1 D S G I D S S H P D L N V R G G A S F V P
64 GACAGCGGA ATTGACTCT TCTCATCCT GACTTGAAC GTCAGAGGC GGAGCAAGC TTCGTACCT

+1 S E T N P Y Q D G S S H G T H V A G T V A
127 TCTGAAACA AACCCATAC CAAGATGGC AGTTCTCAC GGCACACAT GTAGCCGGT ACGGTTGCC

+1 A L N N S I G V L G V A P N A S L Y A V K
190 GCACTTAAT AACTCAATC GGTGTTTTG GGCGTAGCG CCAAACGCA TCGTTATAT GCAGTAAAA

+1 V L D S T G N G Q Y S W I I N G I E W A I
253 GTTCTTGAT TCAACAGGA AACGGCCAA TACAGCTGG ATTATTAAC GGCATTGAG TGGGCCATT

+1 S N K M D V I N M S L G G P S G S T A L K
316 TCCAACAAA ATGGACGTG ATTAACATG AGCCTTGGC GGACCTTCT GGTCTACA GCTTTGAAA

+1 S V V D R A V A S G I V V V A A A G N E G
379 TCAGTCGTT GATAGAGCC GTAGCCAGC GGTATCGTC GTTGTGCT GCAGCCGGA AATGAAGGC

+1 T S G S S S T I G Y P A K Y P S T I A V G
442 ACTTCCGGA AGCTCAAGC ACAATCGGC TATCCTGCA AAATATCCT TCTACCATT GCGGTAGGT

+1 A V N S S N Q R G S F S S V G P E L E V M
505 GCGGTAAAC AGCAGCAAC CAAAGAGGT TCATTCTCA AGCGTAGGT CCTGAGCTT GAAGTCATG

+1 A P G
568 GCTCCTGGC

DNA Sequence 3A13 *B.subtilis* isolate

FIG. 16

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```

      M M R K K S F W L G M L T A L M L V F T M
1  ATGATGAGG AAAAAGAGT TTTTGGCTT GGGATGCTG ACGGCCTTA ATGCTCGTG TTCACGATG

      A F S D S A S A A Q P A K N V E K D Y I V
64 GCCTTCAGC GATTCCGCG TCTGCTGCT CAGCCGGCG AAAAATGTT GAAAAGGAT TATATTGTC

      G F K S G V K T A S V K K D I I K E S G G
127 GGATTTAAG TCGGGAGTG AAAACCGCA TCCGTCAAA AAGGACATC ATCAAAGAG AGCGGCGGA

      K V D K Q F R I I N A A K A K L D K E A L
190 AAAGTGGAC AAGCAGTTT AGAATCATC AACGCGGCA AAAGCGAAG CTAGACAAA GAAGCGCTT

      E E V K N D P D V A Y V E E D H V A H A L
253 GAGGAAGTC AAAAATGAT CCGGATGTC GCTTATGTG GAAGAGGAT CACGTAGCT CATGCTTTG

      A Q T V P Y G I P L I K A D K V Q A Q G Y
316 GCGCAAACC GTTCCTTAC GGCATTTCCT CTCATTAAA GCGGACAAA GTGCAGGCT CAAGGCTAC

      K G A N V K V A V L D T G I Q A S H P D L
379 AAGGGAGCG AACGTAAAA GTCGCCGTC CTGGATACA GGAATCCAA GCTTCTCAT CCGGACTTG

      N V V G G A S F V A G E A Y N T D G N G H
442 AACGTAGTC GCGGAGCA AGCTTCGTA GCTGGCGAA GCTTATAAC ACCGACGGC AACGGACAC

      G T H V A G T V A A L D N T T G V L G V A
505 GGCACGCAT GTTGCCGGT ACAGTAGCT GCGCTTGAC AATACAACG GGTGTATTA GCGTTCGG

      P N V S L Y A V K V L N S S G S G S Y S G
568 CCGAACGTA TCCTTGATC GCGGTTAAA GTGCTGAAT TCAAGCGGA AGCGGATCT TACAGCGGC

      I V S G I E W A T T N G M D V I N M S L G
631 ATTGTAAGC GGAATCGAG TGGGCGACG ACAAACGGC ATGGATGTT ATCAACATG AGCCTTGA

      G P S G S T A M K Q A V D N A Y A R G V V
694 GGACCATCA GGCTCAACA GCGATGAAA CAGGCGGTT GACAATGCA TATGCAAGA GGGGTTGTC

      V V A A A G N S G S S G N T N T I G Y P A
757 GTTGTGGCG GCTGCTGGG AACAGCGGA TCTTCAGGA AACACGAAT ACAATCGGC TATCCTGCG

      K Y D S V I A V G A V D P N S N R A S F S
820 AAATACGAC TCTGTCATC GCAGTTGGC GCGGTAGAC CCTAACAGC AACAGAGCT TCATTTTCC

      S V G A E L E V M A P G A G V Y S T Y P T
883 AGCGTCGGA GCAGAGCTT GAAGTCATG GCTCCTGGC GCAGGCGTG TACAGCACT TACCCAACC

      S T Y A T L N G T S M A S P H V A G A A A
946 AGCACTTAT GCAACATTG AACGGAACG TCAATGGCT TCTCCTCAT GTAGCGGGA GCAGCAGCT

      L I L S K H P N L S A S Q V R N R L S S T
1009 TTGATCTTG TCAAAACAT CCGAACCTT TCAGCTTCA CAAGTCCGC AACCGTCTC TCCAGTACG

      A T Y L G S S F Y Y G K G L I N V E A A A
1072 GCGACTTAT TTGGGAAGC TCCTTCTAC TATGGAAA GGTCTGATC AATGTGAA GCTGCCGCT

      Q *
1135 CAATAA

```

DNA Sequence 5A36 *B.licheniformis* complete
FIG. 17A

DNA Sequence 3A1 *B.subtilis* complete
FIG. 17B

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**Spectinomycin
Resistance (aadA)**

BamH I (1)

PstI (23)

PstI (23)

IS50

AMP

pMODGALSPEC

7057 bp

EcoR I (4983)

IS50

EcoR I (2461)

Galk

FIG. 18

3 2 1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99

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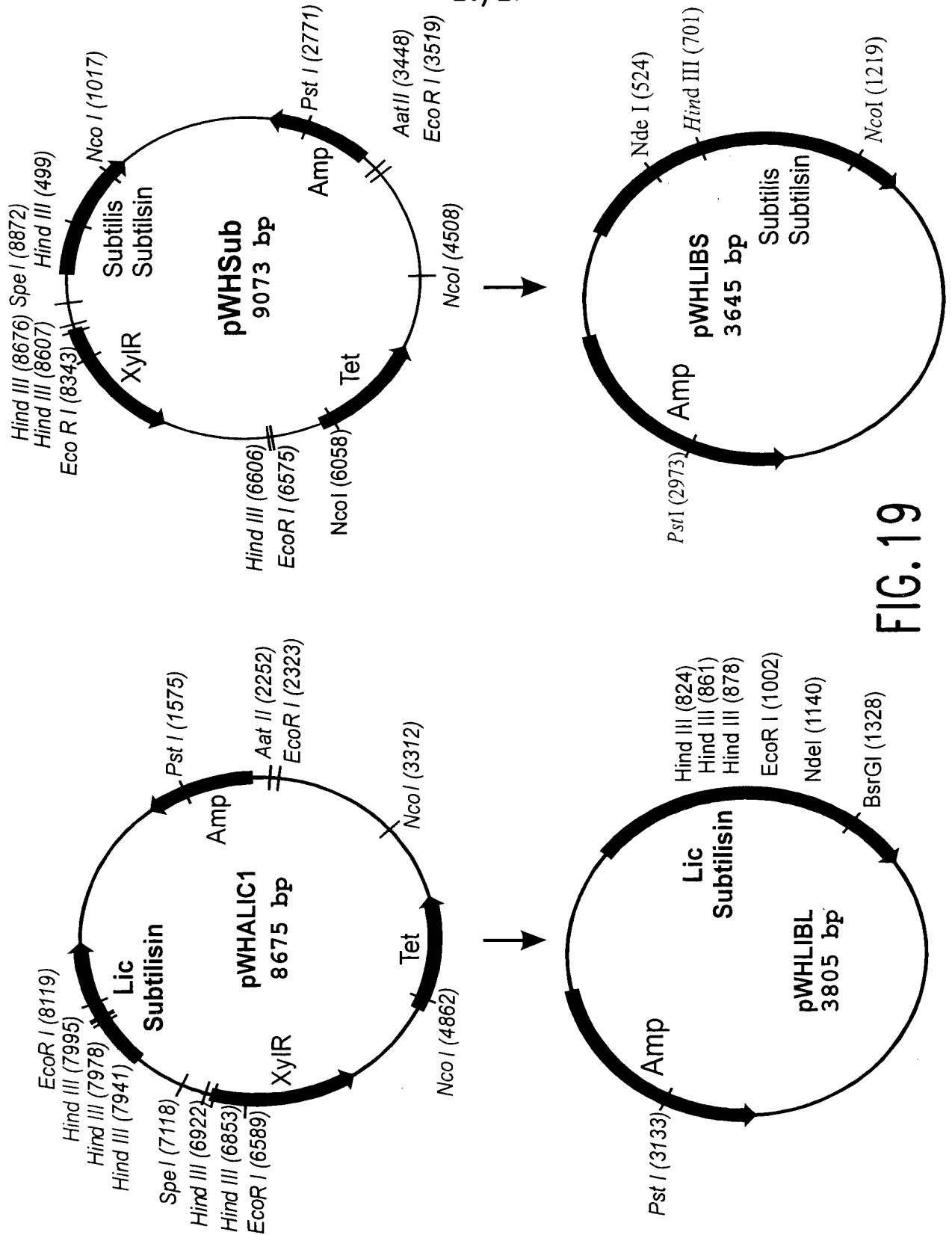


FIG. 19

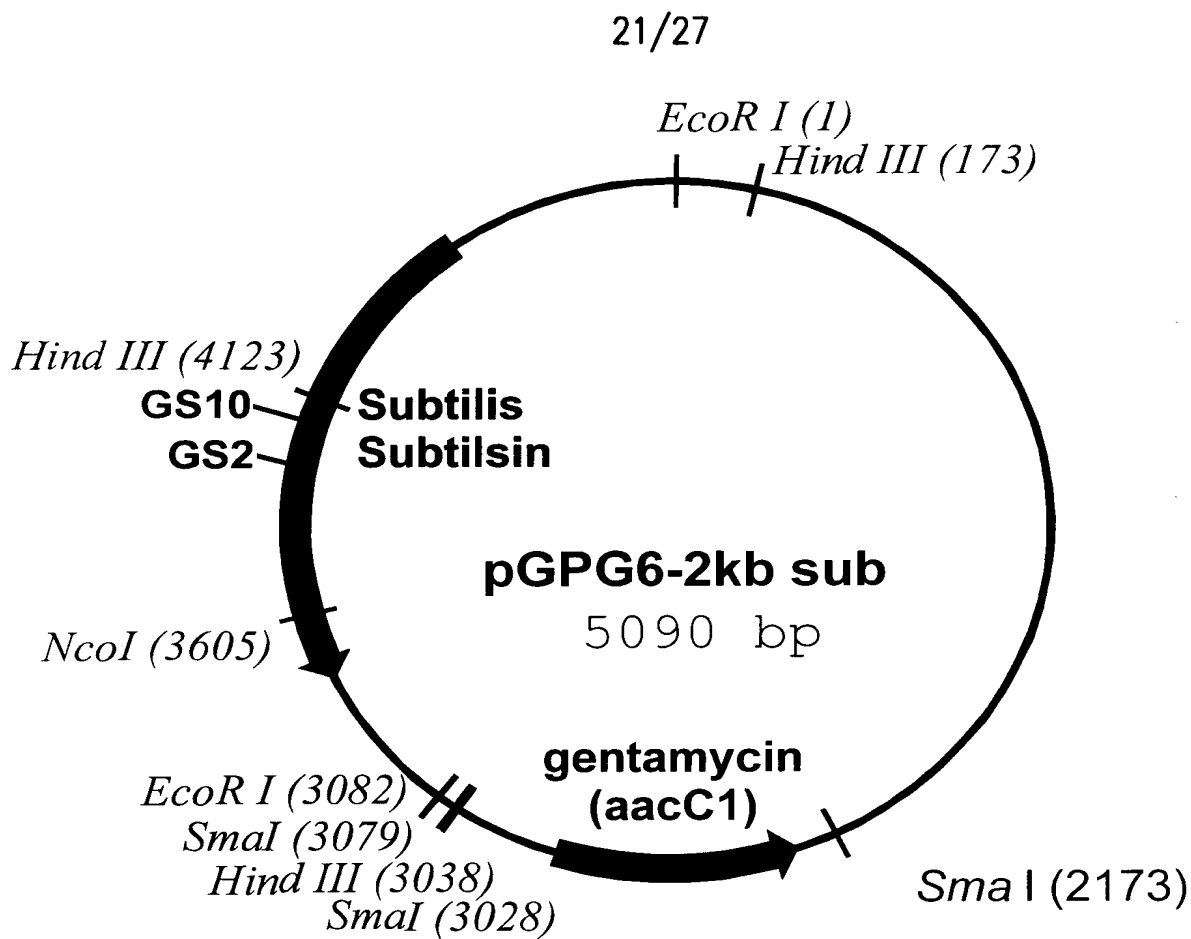
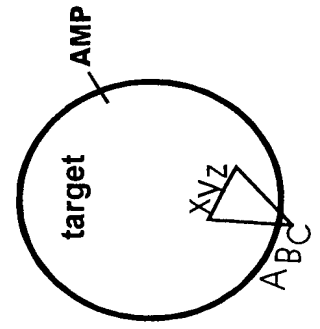


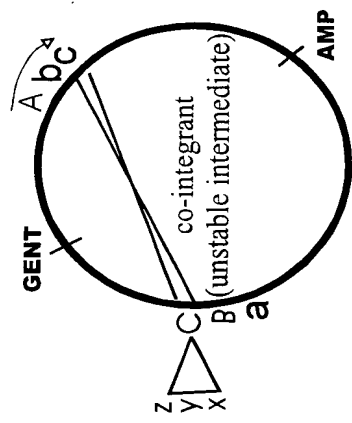
FIG. 20

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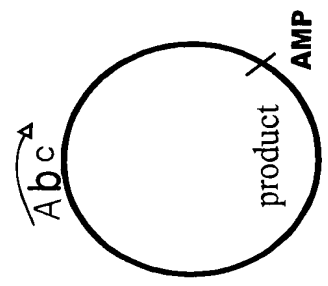
From the co-integrant
possible molecules include:



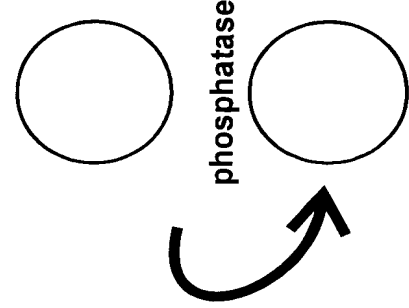
Enzyme digesting
uniquely in the insert (xyz)
will cut



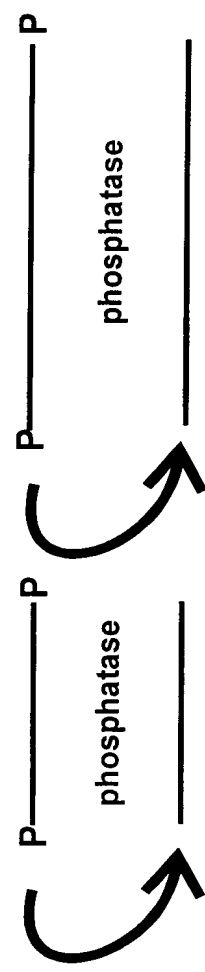
Enzyme digesting
uniquely in the insert (xyz)
will cut



Enzyme digesting
uniquely in the insert (xyz)
does not cut



normal number of
transformants



very few transformants

very few transformants

FIG. 21

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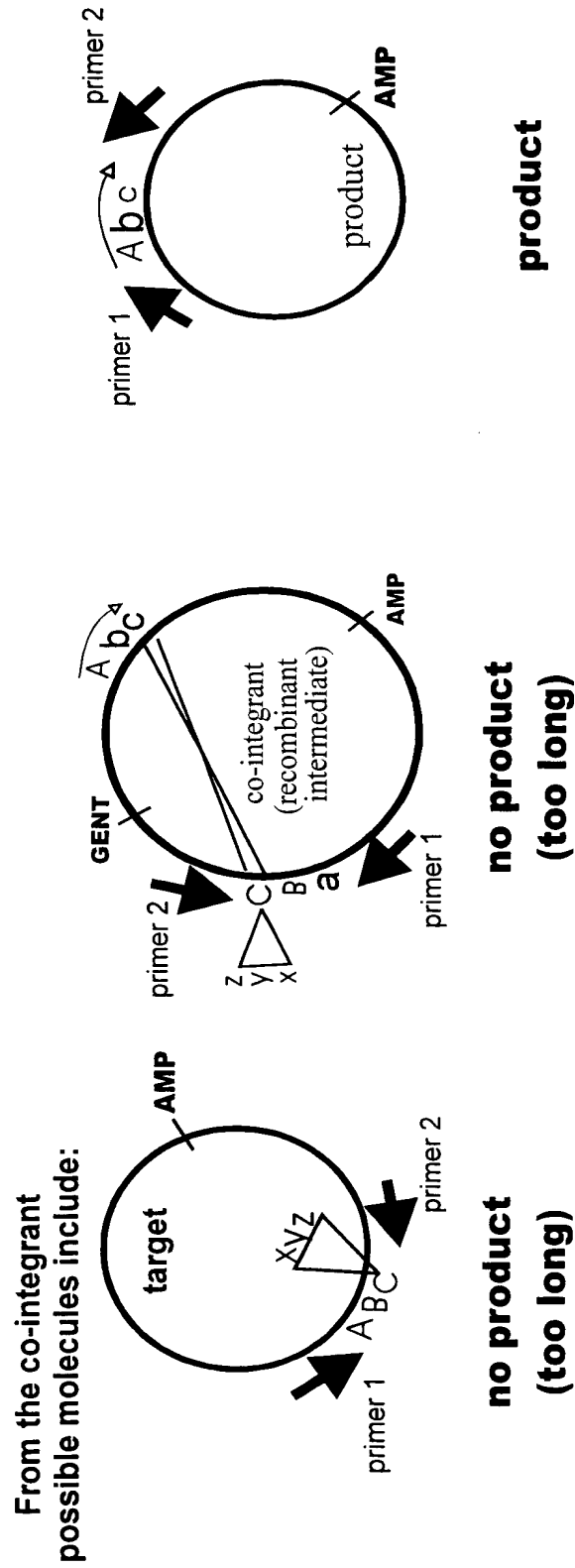


FIG. 22A

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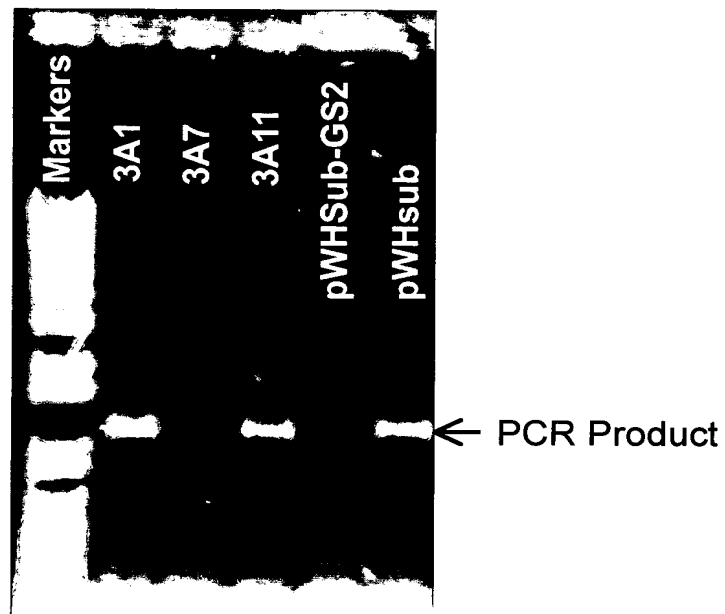


FIG.22B

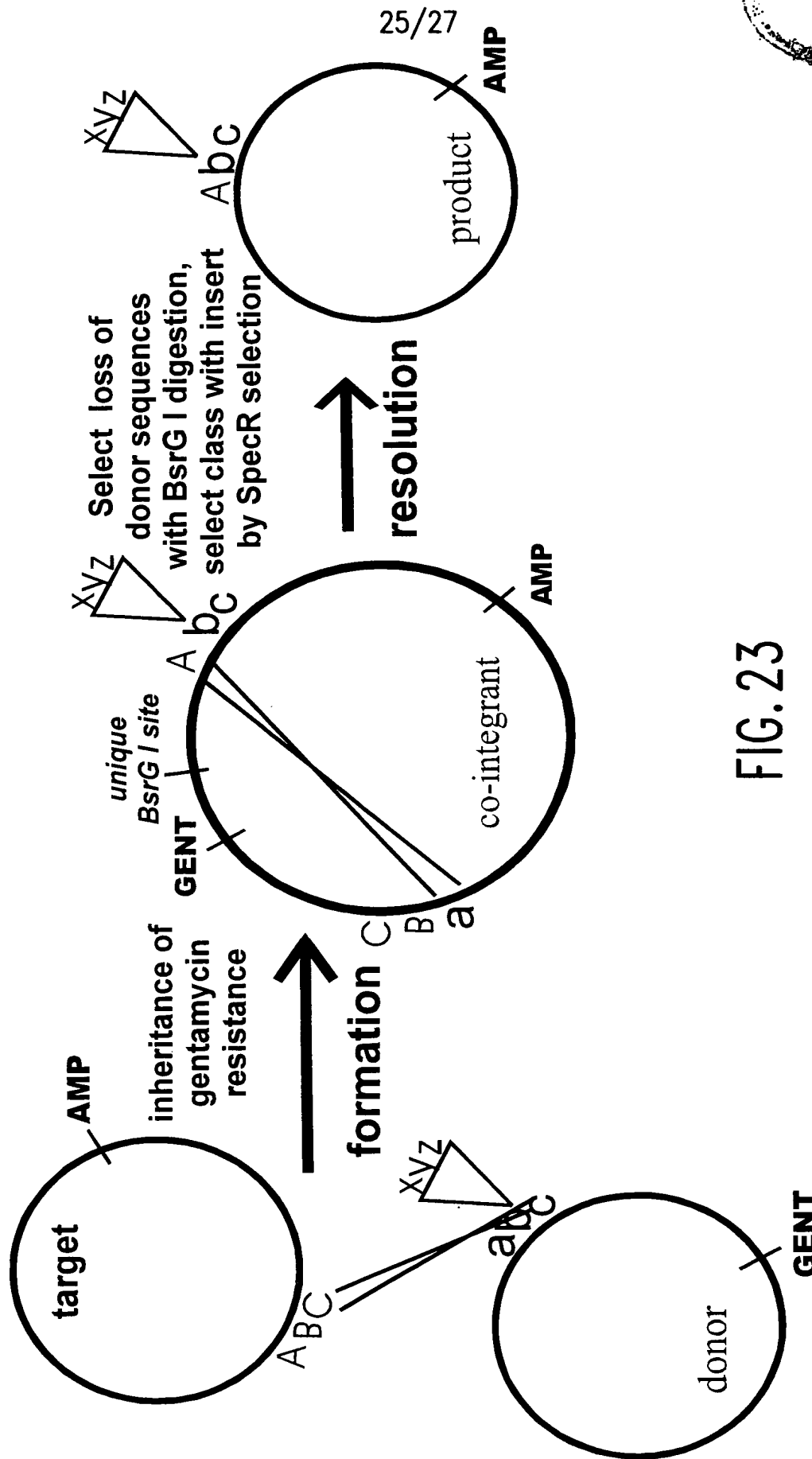
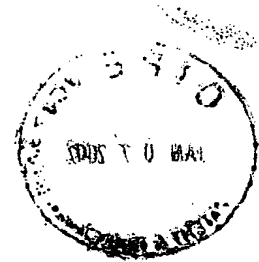


FIG. 23

Oligonucleotide Name	Oligonucleotide SEQ ID NO.	Sequence of oligonucleotide
MCS1F	SEQ ID NO:1	5'-AATTCGGTTTAAACCTTAATTAAGGTACCCATTTTGGCAGATCTAGACCAAAAA TGGGGGGCGCGCTCCCGGGTGGCGGCC-3'
MCS1R	SEQ ID NO:2	5'-AATTGGCGCCACCCGGGAGCGCGCCCAATTTTGGTCTAGATCTGCCAAA AAATGGGTACCTTAATTAAGTTAAACGCG-3'
BglKF	SEQ ID NO:3	5'-GACTGCGAGATCATAGATATAGATTTCACCTACGCGGCTGCTCAAACCTGG-3'
BglKR	SEQ ID NO:4	5'-CCAGGTTTGAGCAGCGCGTAGTGAATCTATATCTATGATCTCGCAGTC-3'
CC_UPPER	SEQ ID NO:5	5'-AATTACCATGGAGCAATGCATATGGTTAAACAGCTCGAGTAGATCTTGGCGCC GCTTGGCTAGCGTCAGCTGGGTACCATGCAT-3'
CC_LOWER	SEQ ID NO:6	5'-CGCGTTATGCATGGTACCCAGCTGACGCTAGCCCAAGCGCGCAAGATCTACTCGAG CTGTTAAACCATATGCAATTGCTCCATGG-3'
internal primer- upper	SEQ ID NO:7	5'-CGCAA(T/A)C(T/C)GTCCTTA(C/T)GG-3'
internal primer- upper	SEQ ID NO:8	5'-GCCAGGAGCCAT(C/G)AC(A/T)TCAA-3'

FIG. 24A

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Oligonucleotide Name	Oligonucleotide SEQ ID NO.	Sequence of oligonucleotide
<i>B. lichenformis</i> Subtilisin Forward Primer	SEQ ID NO:9	5'-GGGTACCGCGTCTATTACATCTTCG-3'
<i>B. lichenformis</i> Subtilisin Reverse Primer	SEQ ID NO:10	5'-GCAGATCTCATTTGTTAGATATGTTATTGACCGGC-3'
<i>B. subtilis</i> Subtilisin Forward Primer	SEQ ID NO:11	5'-AGCGAGATCTCTATTATTGTCAGCTG-3'
<i>B. subtilis</i> Subtilisin Reverse Primer	SEQ ID NO:12	5'-GCGCGGTACCTGATAAAAGGAGAGGGTAAAGAG-3'
Galactokinase upper primer	SEQ ID NO:17	5'-GGAAGATCTAGAGGTTTTCACCGTCATCACCG-3'
Galactokinase lower primer	SEQ ID NO:18	5'-GCTAGATCTCTTTTCGTCGTCCTCAAGAATCCCG-3'

FIG. 24B